Oncogene (2001) 20, 3806–3813

www.nature.com/onc

Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity

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Paclitaxel (PTX), a microtubule-active agent, blocks cell proliferation by inhibiting mitotic progression leading to mitotic and postmitotic arrest and cell death. Here we demonstrate for the first time that very low concentrations of PTX (3–6 nM) can completely inhibit cell proliferation without arresting cells at mitosis. At these low concentrations that are insufficient to inhibit mitotic progression, PTX induced both p53 and p21 causing G1 and G2 arrest in A549. In contrast, low PTX concentrations failed to induce G1 and G2 arrest in A549/E6 cells, that do not express p53. Furthermore, we observed that the levels of p53 and p21 induced by adriamycin and by low concentrations of PTX in A549 cells were comparable. This observation led us to conclude that low concentrations of PTX can induce p53 and p21 sufficiently to cause G1 and G2. Many other cell lines, including HCT116 cells, do not readily upregulate p53 in response to PTX, and therefore undergo exclusively mitotic and postmitotic arrest after PTX treatment. At low concentrations that do not cause mitotic arrest, PTX did not significantly inhibit proliferation of these cells. In HCT116 cells, loss of p53 (HCT/p53$^{-/-}$) or p21 (HCT/p21$^{-/-}$) affects both Bax and Bcl-2 expression. In cells lacking p53, levels of Bax and p21 were decreased. In cells lacking p21, levels of wt p53 were highly increased to compensate for the loss of p21. This in turn results in upregulation of Bax and downregulation of Bcl-2 resulting in an increase of the apoptotic Bax/Bcl2 ratio consistent with increased sensitivity of these cells to apoptotic stimuli. High levels of p53 and Bax/Bcl-2 ratio can also explain why loss of p21 is rarely found in human cancer. *Oncogene* (2001) **20,** 3806–3813.

Keywords: paclitaxel; G1 arrest; mitosis; p53; p21; Bcl-2

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Received 10 January 2001; revised 26 March 2001; accepted 26 March 2001

Introduction

Microtubule-active drugs such as paclitaxel (PTX) inhibit mitotic progression and arrest cells in mitosis (Horwitz, 1992; Rowinsky and Donehower, 1995; Jordan and Wilson, 1998; Blagosklonny and Fojo, 1999). Following mitotic arrest, cells can either undergo apoptosis or exit from mitosis. In the later case, cells with 4 N DNA content are arrested in G1 (pseudo G1). Wt p53 and p21 are involved in postmitotic G1 arrest, whereas G1 checkpoint-deficient cells undergo endoreplication (Cross *et al*., 1995; Fukasawa *et al*., 1996; Khan and Wahl, 1998; Lanni and Jacks, 1998; Motwani *et al*., 2000). Unlike postmitotic arrest, neither mitotic arrest nor apoptosis requires wt p53 (Bhalla *et al*., 1993; Woods *et al*., 1995; Fan *et al*., 1998; Bacus *et al*., 2001). On the other hand, numerous conficting observations suggest that the p53 status affects cell sensitivity to PTX even though the mechanism is unclear (Wahl *et al*., 1996; Wu and El-Deiry, 1996; Safran *et al*., 1996; Gan *et al*., 1996; Debernardis *et al*., 1997; O'Connor *et al*., 1997; Fan *et al*., 1998; Nielsen *et al*., 1998; Vikhanskaya *et al*., 1998; Giannakakou *et al*., 2000a).

It has been demonstrated that low concentrations of DNA damaging drugs such as adriamycin induce p53- and p21-dependent arrest in G1 and/or G2, thus preventing mitotic arrest and the cytotoxicity otherwise caused by microtubule-active drugs (Bunz *et al*., 1998; Blagosklonny *et al*., 2000). In a cell type-dependent fashion, PTX induces wt p53 in A549 and MCF-7 cancer cells and in normal fibroblasts (Blagosklonny *et al*., 1995; Wahl *et al*., 1996; Debernardis *et al*., 1997; Torres and Horwitz, 1998; Zaffaroni *et al*., 1998; Sablina *et al*., 2001). However, PTX only marginally induces wt p53 in many other cell lines including HCT116 cancer cells (Blagosklonny *et al*., 2000). Therefore, two groups of cell lines could be distinguished: (1) with PTX-inducible wt p53 and (2) with wt p53 that does not respond to PTX as well as cells lacking wt p53. Considering this difference as a key determinant, we investigated representative cells lines from each group, namely A549 and HCT116 cells.

Results

Paclitaxel-induced p53 is associated with interphase arrest

Paclitaxel (PTX) inhibits microtubule dynamics (Jordan *et al*., 1996; Jordan and Wilson, 1998) and causes mitotic arrest that is manifested as G2/M (4N-arrest) observable by flow cytometry analysis. In agreement with a previous study (Torres and Horwitz, 1998), we observed by flow cytometry that treatment of A549 cells with PTX at concentrations above 12 nM caused G2/M arrest. Surprisingly, low concentrations of PTX (3–6 nM) inhibited proliferation of A549 cells as potently as higher concentrations of PTX (Figure 1b,c). Similar results were observed with MCF-7 cells (data not shown). We next measured levels of p53 in total cell lysates of A549 cells treated with various concentrations of PTX. We found that wt p53 was induced by PTX with maximal induction at 6–12 nM of PTX (Figure 1d). This was accompanied by induction of p21, a CDK inhibitor. We compared levels of p53 induced by PTX and adriamycin, using both immunoblot and immunochemistry, and found that p53 levels were comparable (Figures 1d and 2). This raised the unexpected possibility that low concentrations of PTX could arrest A549 cells in G1 and/or G2 phases.

As shown in Figure 2, wt p53 is accumulated in the nucleus of interphase cells. Flow cytometry showed that after treatment with 6 nM PTX, cell cycle arrest occurred at multiple points but predominantly at G2/M after treatment with 100 nM PTX (Figure 3 upper panel). Consistent with this, only 7% of cells were arrested in mitosis after treatment with 6 nM PTX, as determined by the mitotic index (Figure 3, lower panel), absence of round mitotic cells (Figure 3 middle panel), and absence of Bcl-2 phosphorylation and histone H3 (Figure 1d). Bcl-2 and histone H3 phosphorylation was detected in mitotic arrest caused by 25–100 nM of PTX (Figure 1d).

At higher concentrations (100 nM), PTX caused predominantly G2/M arrest, as shown by flow cytometry (Figure 3 upper panel). Although the G2/M peak seen after 100 nM PTX seems to be consistent with mitotic arrest, mitotic index (DAPI staining) demonstrated less than 50% of cells arrested in mitosis (Figure 3 lower panel). Live microscopy demonstrated that most cells were attached, and not rounded and detached as mitotic cells (Figure 3). These results were consistant with the absence of histone H3 and Bcl-2 phosphorylation (Figure 1d). We conclude that even at high concentrations of PTX only half of the cells were arrested in mitosis, while the other half were arrested in interphase.

Effects of E6 on PTX sensitivity in A549 cells

To further evaluate the role of p53 in the G1 and G2 arrest, we compared A549 cells expressing an E6 construct (A549-E6) with the cells transfected with an

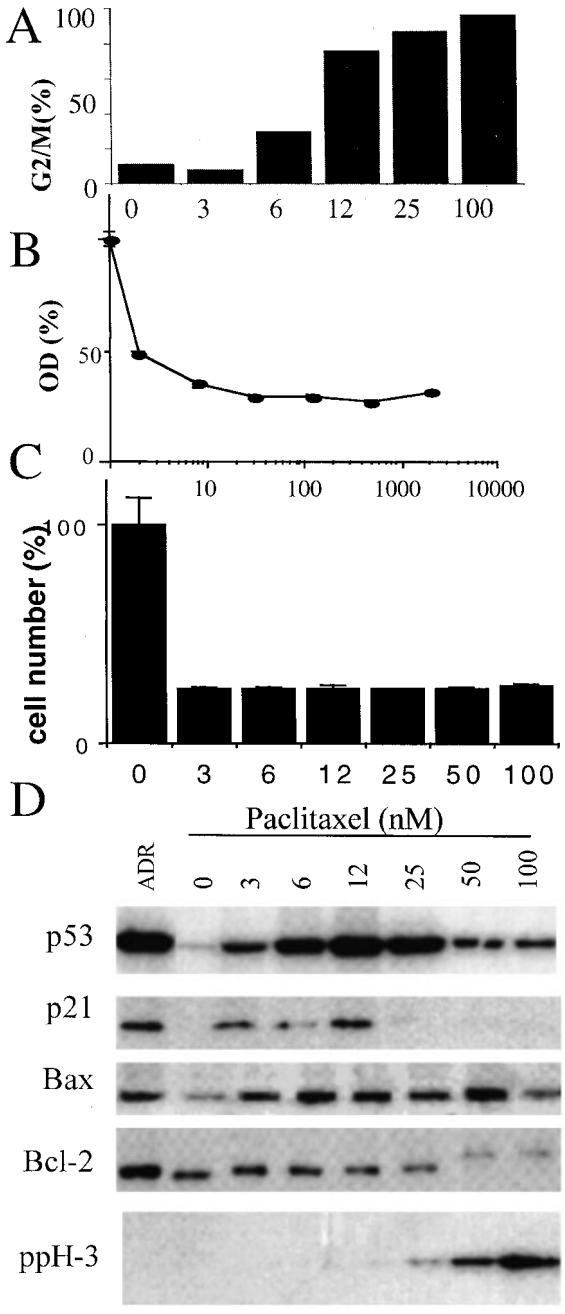

Figure 1 Dose response of PTX in A549 cells. A549 cells were incubated with increasing concentrations of PTX from 3 to 100 nM: (**a**) G2/M phase arrest. After 16 h, cell cycle analysis was performed as described in Materials and methods. Per cents of G2/M cells are shown. (**b**) MTT assay was performed after 3 days as described in Methods. Results calculated as per cent of values of untreated cells and represent mean ± s.d. (**c**) Cell number. After 72 h, cell numbers were counted in triplicate, as described in Materials and methods. Results calculated as per cent of values of untreated cells. (**d**) After 16 h, cells were lysed and immunoblot for p53, p21, Bax and Bcl-2 was performed as described in Materials and methods. Results with 200 nM Adriamycin (ADR) are shown for comparison

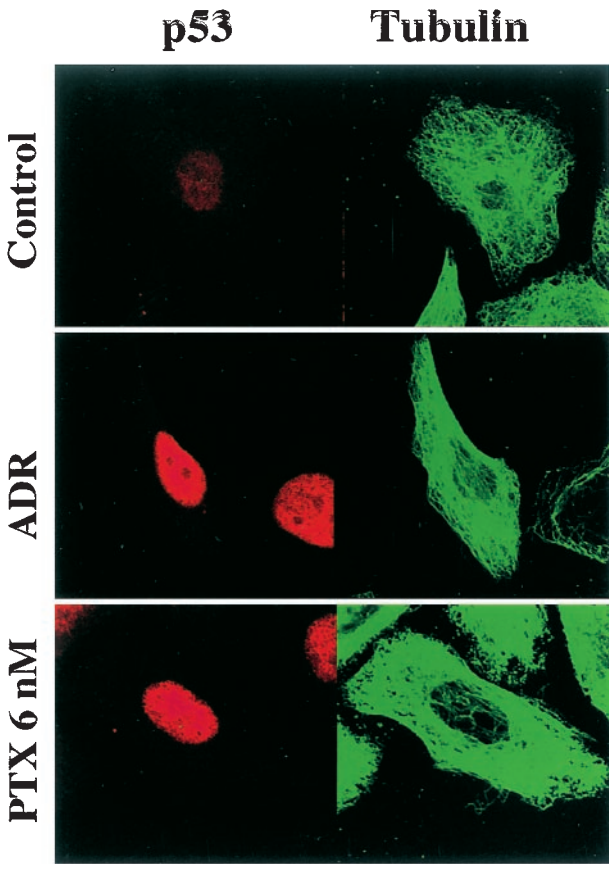

Figure 2 Immunostaining for p53 and tubulin. A549 cells were incubated with 6 nM paclitaxel (PTX 6), 200 nM adriamycin (ADR), or were left untreated (CTRL). After 16 h, cells were fixed and immunochemistry for p53 (red) and tubulin (green) was performed as described in Materials and methods

empty vector (A549-LXSN) (Maurer *et al*., 1999). E6 binds to p53 and targets it for degradation. Consequently, A549-E6 cells have very low levels of the p53 protein (Figure 4a). Low concentrations of PTX (6 nM) resulted in higher induction of p21 in A549-LXSN cells than in A549-E6 cells (Figure 4a). Since E6 partially prevented paclitaxel-induced p53 and p21, we expected partial abrogation of p53-dependent effects in A549-E6 cells. Consistent with this, low doses of PTX induced G1 arrest to a greater extent in A549-LSXN cells compared with A549-E6 cells (Figure 4b). We conclude that G1 arrest caused by low concentrations of PTX depends on induction of p53.

PTX causes exclusively mitotic and postmitotic arrest in HCT116 cells

In HCT116 cells, PTX did not induce wt p53 nor p21 (Figure 5a). At concentrations that induced mitotic arrest (above 12 nM), PTX killed most HCT116 cells by 3 days (Figure 5b). In agreement with a previous study (Long and Fairchild, 1994), HCT116 cells may exit mitosis (mitotic exit) to form multinucleated cells. As shown in Figure 5, a G2/M peak on flow cytometry caused by 100 nM PTX (upper panel) represented exclusively mitotic and post-mitotic cells but not G2 cells (87% mitotic cells by DAPI). Given that mitotic arrest is determinant of the cytotoxicity of PTX, lack of p53 induction renders cells sensitive to mitotic arrest and PTX-induced cytotoxicity. A previous study noted that HCT116 cells lacking p21 are more sensitive to paclitaxel than parental cells (Stewart *et al*., 1999). In contrast, HCT-E6 cells with very low p53 levels were reported as more resistant (Wu and El-Deiry, 1996). Since we did not detect p53/p21 induction in HCT116 cells by paclitaxel, we could not explain why loss of p21 changes the sensitivity to PTX. We chose to investigate the possibility that loss of p21 affects levels of other apoptotic and anti-apoptotic proteins.

Levels of p21, p53, Bcl-2 and Bax are inter-dependent

Wt p53 regulates numerous genes including Bax and p21 (El-Deiry *et al*., 1993; Miyashita and Reed, 1995) which have opposing effects on the cell sensitivity to PTX (increase and decrease sensitivity, respectively) (Strobel *et al*., 1996; Barboule *et al*., 1997; Schmidt *et al*., 2000; De Feudis *et al*., 2000). Furthermore, p53 can selectively induce p21 without induction of Bax and also can downregulate Bcl-2 (Friedlander *et al*., 1996; Ludwig *et al*., 1996; Thornborrow and Manfredi, 1999). We first measured basal and DNA-damage inducible levels of p53, p21 and Bcl-2 (Figure 6). As expected, adriamycin induced p53 and p21 in parental HCT116 cells. However, in p21−/− cells high basal levels of p53 were detected. Basal levels of p53 are determined by a feedback control of its function and loss of p21 could determine, at least in part, increase in p53 levels (Blagosklonny, 1997). This may explain high basal levels of wt p53 and lack of inducibility observed in HCT116 p21−/− cells. Also, both p21-deficient clones (S14 and S4) expressed low levels of Bcl-2. Similarly it has been shown that inhibition of the expression of the antiapoptotic protein Bcl-X-L, resulting in an altered ratio of BAX to BcL-X-L, increased mitochondria-mediated cell death in HCT116 cells (Zhang *et al*., 2000). In contrast, HCT116-E6 have reduced p53, and in turn reduced p21, but express normal levels of Bcl-2 (Figure 6).

Lack of p53 in HCT116-E6 cells and high levels of wt p53 in HCT116 p21−/− resulted in opposing ratios of Bcl-2 and Bax expression (Figure 7a). HCT116-E6 cells are more resistant to PTX (Wu and El-Deiry, 1996) and have a low Bax/Bcl2 ratio (Figure 7a), while HCT116 p21−/− are more sensitive to PTX (Stewart *et al*., 1999) and have a high Bax/Bcl-2 ratio (Figure 6a). In both E6-expressing cells and HCT116 p53−/− cells, levels of p21 and Bax were diminished (Figure 7b).

Discussion

At concentrations lower than those that affect amount of polymerized tubulin, all antimicrotubule drugs

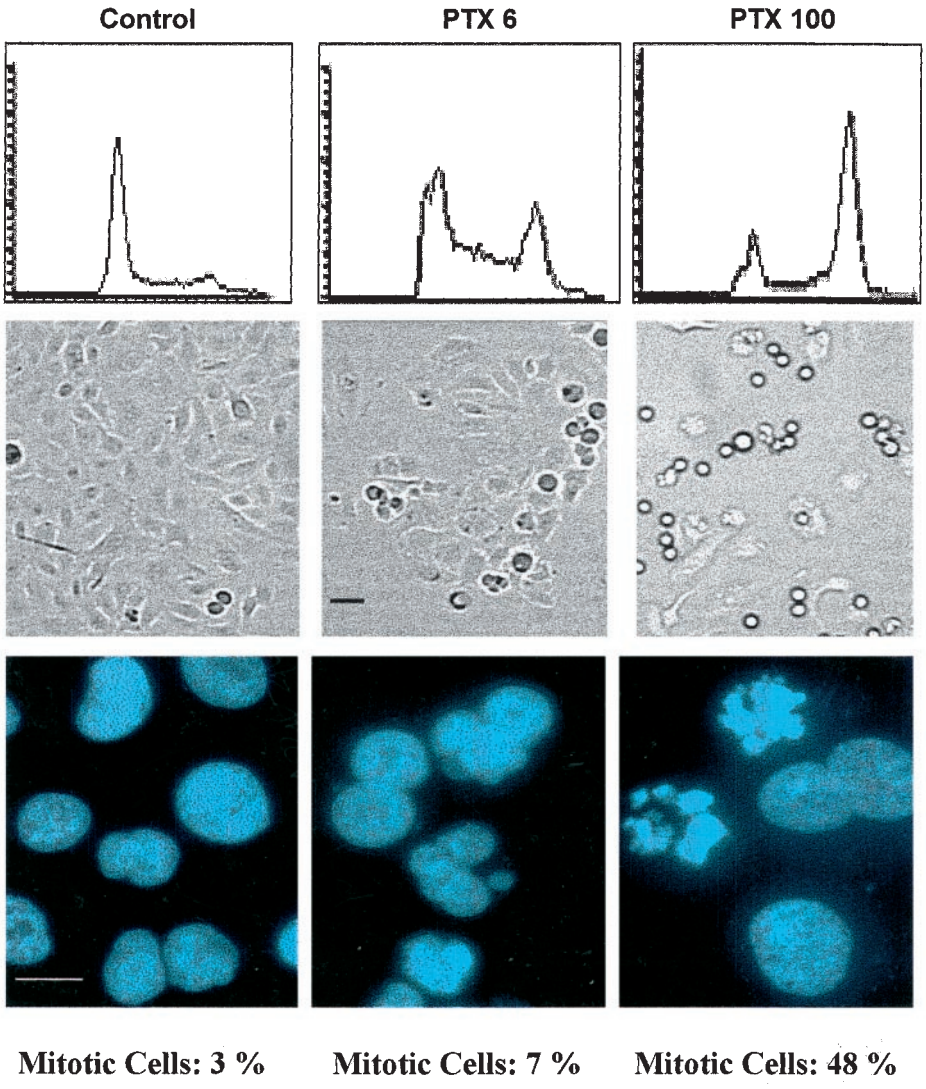

Figure 3 Mitotic versus interphase arrest in A549 cells. A549 cells were incubated with 6 nM PTX, 100 nM PTX, or left untreated, as indicated. Next day, the following assays were performed: (**a**) Flow cytometry was performed as described in Materials and methods; (**b**) Photo microscopy of live culture; (**c**) DAPI staining for mitotic figures and multinucleated cells

suppress functions of mitotic microtubules, leading to mitotic arrest and cell death (Jordan and Wilson, 1998). By flow cytometry analysis, PTX-induced arrest is manifested by 4 N DNA peak (which is often referred to as G2/M) which exclusively represents mitotic and postmitotic cells. Postmitotic G1 arrest depends on p53 and p21 (Cross *et al*., 1995; Fukasawa *et al*., 1996; Khan and Wahl, 1998; Lanni and Jacks, 1998; Motwani *et al*., 2000). Although a primary G1 arrest has been also described, it has been attributed to super-pharmacological concentrations of PTX without any mechanistical explanation (see Blagosklonny and Fojo, 1999). We observed that low concentrations of PTX (3 nM) completely inhibited proliferation of MCF-7 (not shown) and A549 cells (both cell lines with wt p53) without causing mitotic arrest. This growth inhibition was associated with p53 and p21 induction. It is well known that DNA damaging drugs such as adriamycin induce p53 and p21, leading to G1 and/or G2 arrest (Sionov and Haupt, 1999; Vousden, 2000; Vogelstein *et al*., 2000). By comparing p53 and p21 levels induced by adriamycin and by low doses of PTX, we conclude that levels of PTX-induced p53 can be sufficient to cause G1 and G2 arrest. Low concentrations of PTX preferentially arrested A549 cells in G1 and G2. Although 100 nM PTX caused 100% of A549 cells to accumulate in G2/M as assessed by flow cytometry, only half of G2/M cells were arrested in mitosis while the remaining cells were arrested in G2 and in G1 phases with 4N DNA content (multinucleated cells). Both low and standard concentrations of PTX were equally cytostatic by inhibiting cell cycle progression regardless of the point of cycle arrest. It is noteworthy that p21-mediated arrest can be

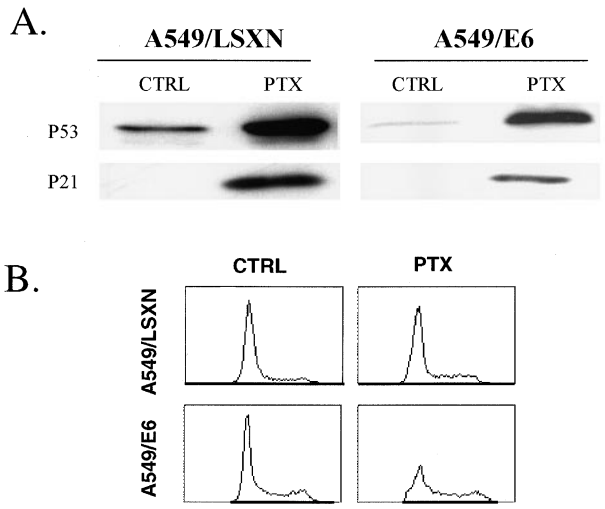

Figure 4 Comparison of A549-E6 and A549-LXSN cells. (**a**) A549/LXSXN and A549/E6 cells were incubated with or without 3 nM PTX for 16 h, and then immunoblot for p53 and p21 was performed as described. Overexposed film is shown for A549/E6 cells (**b**) A549/LXSXN and A549/E6 cells were incubated with or without 3 nM PTX for 16 h, and then cell cycle analysis was performed as described in Materials and methods

protective against paclitaxel cytotoxicity due to prevention of mitotic arrest (Yu *et al*., 1998; Li *et al*., 1999; Blagosklonny *et al*., 2000; Schmidt *et al*., 2000). Furthermore, low doses of paclitaxel induced G1 arrest in A549-LSXN cells (transfected with a mock vector) but not in A549-E6 cells.

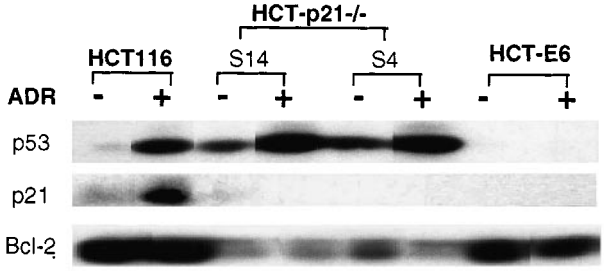

Figure 6 P53 and p21 status of HCT-p21−/− and HCT-E6 cells. HCT116, HCT-p21−/− (S14, S4), HCT-E6 cells were incubated without or with 200 nM adriamycin (ADR: ±) for 16 h, and then immunoblot for p53, p21, and Bcl-2 was performed as described in Materials and methods

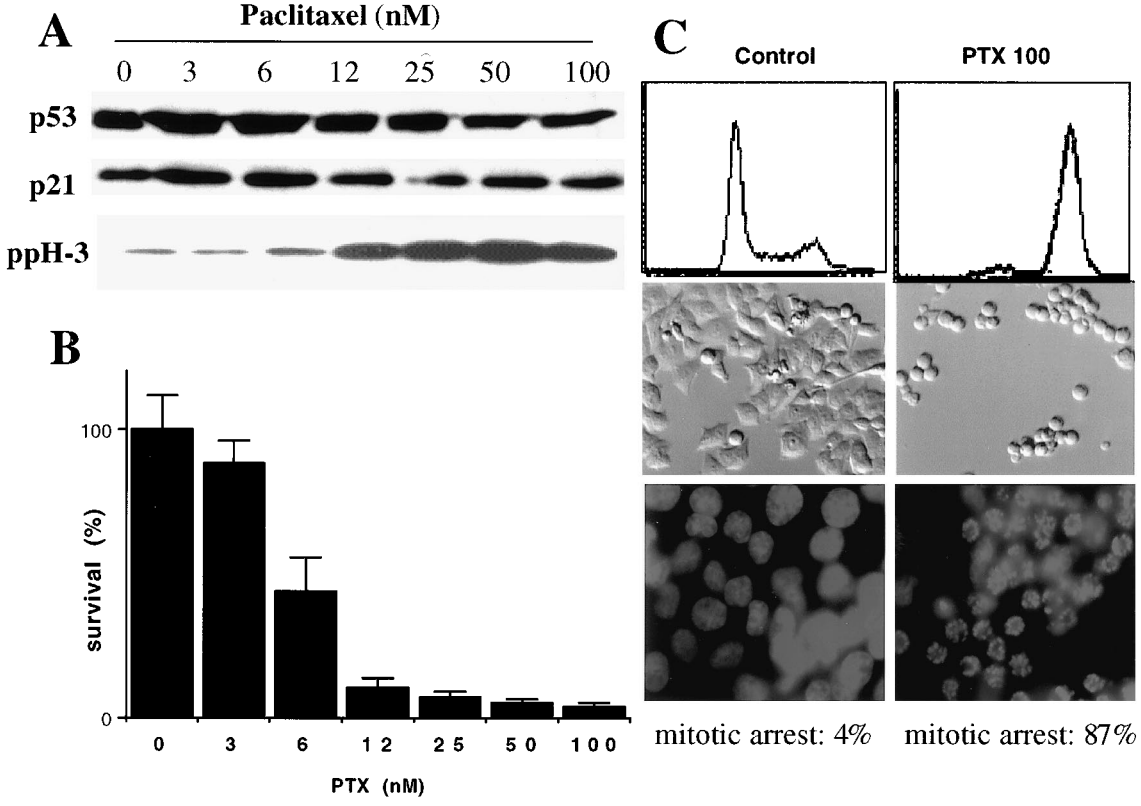

Figure 5 Lack of p53 induction and mitotic arrest in HCT116 cells. (**a**) HCT116 cells were incubated with indicated concentrations of PTX for 16 h, and then immunoblot for p53 and p21 was performed as described. (**b**) HCT116 cells were incubated with indicated concentrations of PTX for 3 days, and then number of live cells was counted in triplicate as described. Results calculated as per cent of values of untreated cells and represent mean of three experiments. (**c**) HCT116 cells were incubated with or without 100 nM PTX and after 16 h cell cycle analysis (upper panel), live cell microphotoscopy and DAPI staining for mitotic cells (low panel) were performed as described in Materials and methods

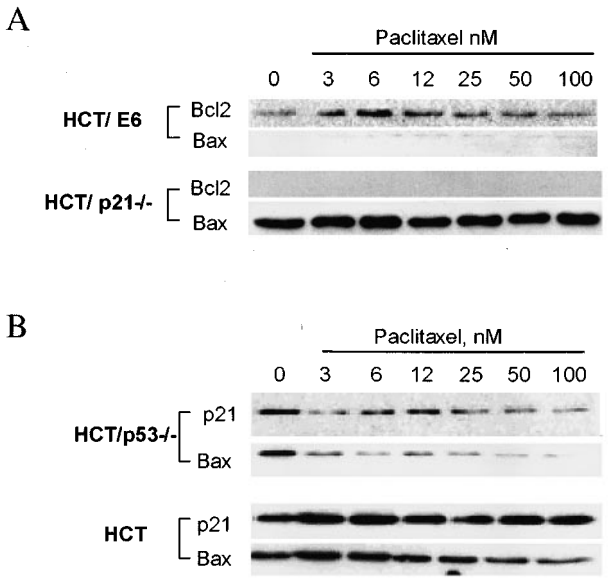

Figure 7 Loss of p53 or p21 affects Bax and Bcl-2 ratios (**a**) Inverse correlation between Bcl2 and Bax levels in HCT-p21−/− and HCT-E6 cells. HCT-p21−/− and HCT-E6 cells were incubated with increasing concentrations of paclitaxel, and then immunoblot for Bcl-2 and Bax was performed as described in Materials and methods. (**b**) Correlation between Bax and p21. HCT-p53−/− and HCT-116 cells were incubated with increasing concentrations of paclitaxel, and then immunoblot for p21 and Bax was performed as described in Materials and methods

This can explain previous observations that PTX induces a late-G1 block in normal fibroblass, but not in T antigen-transformed cells (Trielli *et al*., 1996). PTX selectively induces death of transformed cells, possibly because they escape the PTX-dependent G1 arrest, as well as the G2/M arrest, which are both specific to nontransformed cells (Trielli *et al*., 1996). Importantly, T antigen inactivates p53 (Lane and Crawford, 1979; Linzer and Levine, 1979). We suggest that p53 induction after PTX treatment results in G1 arrest and leads to cytoprotection of nontransformed cells. Similarly, pretreatment with different cytostatics prevents PTX-treated cells from entering mitosis and therefore decreases the cytotoxicity of PTX (Yu *et al*., 1998; Blagosklonny *et al*., 2000; Schmidt *et al*., 2000; Koutcher *et al*., 2000; Zeng *et al*., 2000). Here we showed that the induction of p53 by very low concentrations of PTX can also induce premitotic arrest and completely inhibits cell proliferation.

However, PTX does not induce p53 in many cell types. As illustrated herein, PTX did not induce p53 and p21 in HCT116 cells, and therefore did not prevent mitosis and mitotic arrest in these cells. It has been shown that at latter time points, as a consequence of mitotic arrest and slippage, PTX induced p53 and p21 even in HCT116 cells, thus preventing DNA endoreplication (Stewart *et al*., 1999). p21-deficient HCT116 cells (p21−/− cells) are more sensitive to PTX-induced cell death (Stewart *et al*., 1999). We found that levels of wt p53 were increased in p21−/− cells. An increase of p53 in p21−/− cells can be explained by the fact that cells attempt to compensate for the loss of p21 by increasing p53 levels (Blagosklonny, 1997). In turn, p53 may induce Bax and can decrease Bcl-2 (Miyashita and Reed, 1995). In agreement, we found high levels of Bax and low levels of Bcl-2 in p21-deficient cells. In contrast, loss of p53 in HCT p53−/− cells and in HCT116-E6 cells resulted not only in decreased p21 but also in decreased Bax. The ratio of Bax/Bcl-2 was decreased in p53 null cells and was not affected by PTX exposure. Each of these proteins may affect the drug sensitivity, Bcl-2 and p21 protect cells from paclitaxel while Bax sensitizes cells to PTX (Strobel *et al*., 1996). Therefore, the outcome of the loss of p53 may depend on cell line specific balance of these opposing factors.

Materials and methods

Cell lines and reagents

A549, a human lung cancer cell lines, and MCF-7, a breast cancer cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). A549-E6, E6-transfected A549 cells, and A549-LXSN, A549 cells transfected with an empty vector, were provided by Dr. Cornwell (Fred Hutchinson Cancer Center, Seattle, USA). The human colon cancer cell line HCT116, and two clones lacking p21 (p21−/−), S4 and S14, generated from HCT116 cells (Waldman *et al*., 1995) as well as HCT116 cells lacking p53, HCT116 p53−/− (Bunz *et al*., 1998, 1999) were a gift from Dr B Vogelstein (John Hopkins University). HPV-E6 expressing clones of HCT116 cells, HCT-E6, were a gift from Dr WS El-Deiry (University Pennsylvania, Philadelphia, PA, USA). Paclitaxel (Taxol®), was a Bristol-Myers product (Bristol-Myers, Princeton, New Jersey, USA). Adriamycin was obtained from Sigma (St. Louis, MO, USA) and dissolved in DMSO as a 2 mg/ml stock solution.

Immunoblot analysis

Cells were lysed and soluble proteins were harvested in TNES buffer (50 mM TrisHCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1% (v/v) NP40) containing protease inhibitors (20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM PMSF). Proteins were resolved on 7–12% SDS–PAGE as previously described (Giannakakou *et al*., 2000a). Immunoblot was performed using the following antibodies: antihuman monoclonal WAF1 (EA10), p53 (PAb2) and Bax antibodies (Oncogene Res., Calbiochem, Cambridge, MA, USA), antihuman Bcl-2 monoclonal (DAKO Corp. Carpinteria, CA, USA) antibodies, anti-phospho-Histone H3 rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY, USA).

MTT assay

Two thousand cells were plated in 96-well flat bottom plates and then exposed to the pharmacological agents. After 3 days, 20 μl of 5 mg/ml MTT solution in PBS was added to each well for 2 h. After removal of the medium, 170 μl of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a

Biokinetics plate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). Triplicate wells were assayed for each condition and standard deviations were determined (An *et al*., 2000).

Number of dead and live cells

Cells plated in 24-well plates in 1 ml of medium, or in 96-well plates in 0.2 ml, were treated with the drugs. After the indicated time, cells were counted in triplicate on a Coulter Z1 cell counter (Hialeah, FL, USA). In addition, cells were incubated with trypan blue and the numbers of blue (dead) cells and transparent (live) cells were counted in a hemacytometer.

Cell cycle analysis

Cells were harvested by trypsinization, washed with PBS and resuspended in 75% ethanol in PBS and kept at 4°C for at least 30 min. Prior to analysis, cells were washed again with PBS and resuspended and incubated for 30 min in a solution containing 0.05 mg/ml propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton-X-100 and 1 mg/ml RNAse A in PBS. The suspension was then passed through a nylon mesh filter and analysed on a Becton Dickinson FACScan.

Immunofluorescence

Cells were plated onto glass coverslips in 24-well plates. The following day, cells were treated with PTX or Adriamycin for 16 h, then washed with PBS and fixed with 3% paraformaldehyde for 30 min at room temperature followed by 5 min incubation at −20°C with ice-cold methanol. Cells were then immunostained with an anti-p53 antibody (Ab2 from CalBiochem) or anti-a-tubulin mouse monoclonal antibody (DM1), followed by secondary FITC-conjugated anti-mouse antibody (Vector Lab., Lincolnshire, IL, USA) or Rhodamine RedX anti-mouse antibody (Jackson Immunochemicals, West Grove, PA, USA), respectively. Coverslips were mounted onto glass slides and analysed with a Zeiss LSM 510 microscope as previously described (Giannakakou *et al*., 2000b).

Mitotic index

Cells were incubated with drugs for the indicated time. Cells were washed with PBS, pelleted onto glass slides in a cytocentrifuge, fixed with 90% ethanol/10% glacial acetic acid and stained with DAPI as described previously. Nuclei were visualized by UV microscopy (Blagosklonny *et al*., 2000).

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